

Histamine in Rheumatoid Arthritis

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Abstract

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by a persistent inflammation of the synovium, leading to the erosion of articular cartilage and bone. Synovial mast cells and their effector molecule, histamine, receive increased attention as mediators of joint inflammation. The aim of our study was to analyse levels of free histamine in serum and joint fluid of RA patients and to evaluate the potential inflammatory properties of histamine *in vivo* and *in vitro*. Histamine levels were measured by an ELISA in synovial fluid and sera of RA patients and of healthy controls. Histamine levels were also assessed in plasma of RA patients undergoing anti-TNF- α treatment. In the murine part of the study, histamine was injected intra-articularly in the knee joint of mice and the joints were subsequently analysed with respect to induction of inflammation. RA patients displayed significantly lower levels of histamine in circulation (0.93 ± 0.16 ng/ml) compared with the healthy controls (1.89 ± 0.45 ng/ml, $P < 0.001$). Locally, in synovial fluid the levels of histamine were even lower (0.37 ± 0.16 ng/ml, $P < 0.0006$). Long-term anti-TNF- α treatment significantly increased circulating levels of histamine in RA patients. Our experiments on animals show that histamine on its own neither induces inflammation in the joint cavity nor influences the course of HMGB1 and peptidoglycan-induced joint inflammation. Based on our experimental and clinical studies we suggest that histamine lacks harmful properties in RA.

Introduction

Rheumatoid arthritis (RA) is a disease characterized by a chronic inflammation of the synovium, leading to the erosion of articular cartilage and bone [1–3]. The pathophysiological process of RA is often assigned to the actions of fibroblast-like synoviocytes, macrophages, T lymphocytes and their soluble products, such as pro-inflammatory cytokines and metalloproteinases [4–7].

The role of mast cells (MC) in the pathogenesis of RA is largely unknown; yet circumstantial evidence indicates that MC may play a role in the disease process. An increase in the number of MC was noted in the synovial tissue and fluid of patients with RA [3]. It has also been established that a considerable number of synovial specimens displayed MC activation and degranulation at the site of cartilage erosions [6]. Presence of degranulated MC in joint tissue, before any obvious clinical or histological inflammation, suggests a role for MC in the effector phase of inflammatory arthritis [9]. However, the true role of this cell population in the pathogenesis of RA remains unclear.

Histamine is produced by a wide range of cells that are capable to home from the blood to the synovium. However, circulating blood histamine originates almost entirely from basophiles and from MC [3, 10, 11]. Recently, attention has been turned to histamine and its potentially harmful role in RA. There is evidence that histamine is present in both the diseased synovium and the joint fluid [3, 8, 12–14]. It is, furthermore, believed to be synthesized by an array of leukocytes including T lymphocytes, macrophages and neutrophils, and has been proposed to regulate their cytokine production [4, 5, 15]. Histamine has also been detected in articular cartilage of RA patients, which suggests chondrocytes as another synthesis locus. In addition, the expression of histamine and histidine-decarboxylase (HDC) have been observed in many different tissues of the human body, which suggests that histamine has important functions in the regulation of basic biological cell processes [4, 5, 11].

Histamine acts through four different types of receptors (H₁, H₂, H₃ and H₄) on various cell types. In the human joint, H₁ and H₂ receptors are present on the synovial fibroblasts, as well as on articular chondrocytes,

and they exert their effect through two different pathways [16, 17]. This might be one of the reasons why earlier studies concerning histamine, its presence and role in joints have been contradictory. These findings led to the suggestion that histamine increases inflammation in RA and that presence of histamine in synovial fluid (SF) could be a sign of the disease [13, 18, 19]. On the other hand, some studies have emphasized the anti-inflammatory role of histamine through its capacity to switch a Th1 response to a Th2 response, via binding to H2 receptors, and hence upregulating the secretion of anti-inflammatory cytokines, such as IL-4 and IL-10 [20–23]. This is further supported by a study by Garacz *et al.* [23], where HDC knockout mice (HDC^{−/−}) displayed significantly higher increase of ear swelling when compared with wild-type mice, suggesting that histamine might have a suppressive effect on the production of Th1 cytokines and, consequently, on the inflammatory response. Finally, a growing number of studies suggest that histamine is involved in the downregulation of human polymorphonuclear leukocyte functions in inflammatory responses, such as production of oxygen radicals, cytokine and leukotriene production as well as chemotaxis [24, 26].

In vivo experiments have shown that lymphocytes from RA patients spontaneously release considerably more histamine than do those from healthy blood donors. Thus, the local release of histamine by lymphocytes and MC at the level of the inflamed joint could be a potential source of this molecule [13]. Several studies concerning levels of histamine in plasma, serum and SF in patients with diverse arthritides have been carried out in the past, with conflicting conclusions regarding the impact of histamine on disease severity [3, 18, 19]. The aims of the present study included: (a) simultaneous measurements of histamine levels in serum/joint fluids in well characterized patients with RA; (b) to study prospectively the impact of efficient anti-rheumatic treatment in circulating histamine levels; and (c) to assess *in vivo* and *ex vivo* the potential pro-inflammatory/arthrogenic role of histamine.

Materials and methods

Patients and controls. Sera and synovial fluid samples were collected from 44 patients with RA (16 males, 28 females, age range 25–84 years) who attended the Rheumatology clinics, at Sahlgrenska University Hospital in Gothenburg, Sweden, for acute joint effusion. RA was diagnosed according to the American College of Rheumatology criteria. The study was approved by the Ethics Committee of the University of Göteborg (S-441-01). Clinical characteristics of RA patients are presented in Table 1. At the time of synovial fluid and blood sampling most of the patients received non-steroidal anti-

Table 1 Clinical characteristics of patients with rheumatoid arthritis.

	Erosive RA, n = 34	Non-erosive RA, n = 10
Gender (F/M)	21/13	7/3
Age (years)	61.1 ± 2.3	63.7 ± 5.4
Rheumatoid factor (+/−)	32/2	4/6
Disease duration	14.2 ± 1.5	7.3 ± 2.5
Treated with DMARD (%)		
Methotrexate	20 (59)	2 (20)
Other	6 (18)	1 (10)
Non-treated	8 (24)	7 (70)

inflammatory drugs. Recent radiographs of the hands and feet were obtained for all the patients. Presence of bone erosions, defined as the loss of cortical definition at the joint, was recorded in proximal interphalangeal, metacarpophalangeal, carpal, interphalangeal and metatarsophalangeal joints of forefeet. Presence of single erosion was sufficient to fulfil the requirement of an erosive disease. Presence of the rheumatoid factor (RF) of any of immunoglobulin isotypes was considered as positive. Paired blood samples were also collected from 10 RA patients (eight females and two males, age range 24–72 years, disease duration: 0–23 years, methotrexate dose 7.5–22.5 mg/week) before and after five infusions of antibodies against tumour necrosis factor-alpha (anti-TNF- α , infliximab, total dose 1000 mg). Disease activity score (DAS) and C-reactive protein (CRP) in serum were assessed at each sampling occasion.

Plasma samples from 33 healthy individuals (two males and 35 females, age range 54–67) were used in the control group. Synovial fluid from 19 patients (11 males and eight females, age range 21–43) with traumatic knee injuries was used as a second control group.

Measurement of histamine levels. A sandwich ELISA (ImmunoTech, Marseille, France) was used to assess histamine levels in plasma and SF samples, according to manufacturer's recommendations. In brief, principle of this ELISA is based on a competition-type assay. Histamine of the samples, standards and controls is chemically modified during an acetylation step using *N*-hydroxysuccinimide ester coupled to succinyl-glycaminid (SGA), subsequently forming a histamine-SGA complex. After incubation of the samples, the wells are washed and the bound enzymatic activity is detected by the addition of a chromogenic substrate. The intensity of the colour development is inversely proportional to the histamine concentration in the sample. The absorbance is read at 405 nm. The histamine concentration in samples was calculated by interpolation from a standard curve that is performed in the same assay as the samples. A computer program (DeltaSoftTM) was used to construct a four-parameter curve fit and histamine concentration in the samples was calculated based on that curve.

Intra-articular injection of histamine, HMGB1 and peptidoglycan. To assess pro-inflammatory properties of histamine, 24 healthy female NMRI mice were injected intra-articularly (i.a.) in the knee joint with a total volume of 20 μ l containing 10, 1, 0.1 or 0.01 ng of histamine (Sigma, St Louis, MO, USA) in physiological saline. Control mice were also injected i.a. with the vehicle solution alone. After 6 and 72 h, three mice from each group were sacrificed and the knee joints were obtained for histological analyses. To evaluate potential anti-inflammatory effect of histamine, healthy NMRI mice were injected i.a. with a mixture of histamine (10 ng/knee) and (a) HMGB1 (2 μ g per knee, $n = 16$); or (b) staphylococcal peptidoglycan (10 and 50 μ g per knee, $n = 16$). Control mice were injected i.a. with HMGB1 ($n = 16$) or peptidoglycan ($n = 16$) alone. The mice were sacrificed after 72 h and the knee joints were obtained for histological analyses.

The animal study was approved by the Council for Animal Experiments of the University of Göteborg (271-2003).

Histological examination of knee joints. A histological examination of the knee joints was performed after fixation, decalcification and paraffin embedding of specimens. Tissue sections were prepared and stained with haematoxylin and eosin. Slides were examined and scored with respect to severity of synovitis as follows: 0 = no synovitis, 1 = mild synovitis, 2 = moderate synovitis and 3 = severe synovitis. All evaluations were performed on coded slides in a blinded manner.

Preparation and stimulation of spleen cultures. Mononuclear cell cultures were aseptically obtained from mouse spleens and resuspended in Iscove's complete medium (10% fetal calf serum, 5 \times 10⁻⁵ M 2-mercaptoethanol, 4 mM L-glutamine, 50 μ g gentamycin/ml) at final concentration 1 \times 10⁶ cells per ml. The splenocytes were exposed to histamine at concentrations 0–100 μ M. To evaluate anti-inflammatory properties of histamine, cell cultures were subjected to histamine treatment (10 μ g/ml) overnight and further stimulated with 3 μ g/ml of toxic shock syndrome toxin 1 (TSS-T1), or 1.5 μ g/ml of concanavalin A (Con A) (ICN Biomedicals Inc., Aurora, OH, USA). The supernatants were collected following 24 h stimulation and stored at –20 °C.

Analysis of interleukin-6 and interleukin-10. Interleukin-6 (IL-6) levels in the supernatants were measured by a bioassay based on murine hybridoma cell line B13.29, subclone B9, as described previously. This cell line is dependent on exogenously supplied IL-6 for its growth. Interleukin-10 measurement was performed in plasma from anti-TNF- α -treated RA patients. An analysis was carried out with a sandwich ELISA (BioSite, Täby, Sweden), as recommended by the manufacturer.

Induction of T cell/macrophage and of granulocyte-mediated inflammatory responses in vivo. Granulocyte-mediated and

T-lymphocyte-independent inflammation was induced in mice by injection of 30 μ l of olive oil subcutaneously (s.c.) in hind left paw [27]. Paw swelling was registered with an Odinst spring caliper (Kroplin, Hessen, Germany) before and 24 h after the injection. The inflammatory response was expressed as the increase in paw thickness in 10^{–3} cm. To study T-cell/macrophage-dependent inflammation, mice were sensitized by epicutaneous application of 150 μ l of a mixture of 95% ethanol and acetone (3:1) containing 3% 4-ethoxy-methylene-2-phenyloxazolone (OXA) (Sigma) on the abdomen skin [28]. Seven days after the sensitization all mice were challenged by topical application of 15 μ l of 1% OXA dissolved in acetone and olive oil on both sides of the right ear. The thickness of the ear was measured before and 24 h after challenge using an Odinst spring caliper. The intensity of delayed-type hypersensitivity (DTH) reactions was expressed as (ear thickness 24 h after challenge – thickness before challenge) \times 10^{–3} cm units. Two weeks after the initiation of OXA-induced inflammation, mice were bled and serum was stored at –20 °C. Levels of anti-oxazolone antibodies were measured using an ELISA.

Pre-treatment with sodium cromoglycate. One day prior to sensitizing the mice with OXA or injection with olive oil, eight male NMRI mice were treated intraperitoneally with 200 μ l of sodium cromoglycate (SC, 7.7 mg/ml in PBS) (Lomudal®, Aventis Pharma AB, Stockholm, Sweden), which is an MC membrane-stabilizing agent. This treatment regime has been previously shown to efficiently reduce the levels of histamine in mice [29]. Another 10 mice served as controls and received the vehicle solution.

Analysis of oxazolone antibodies in sera. Ninety-six-well microplates (NUNC, Roskilde, Denmark) were coated over night at 4 °C with OXA conjugated to dog albumin (DSA-OXA) (0.005%) dissolved in PBS.

After washing three times in PBS and blocking with 0.5% BSA (Sigma) dissolved in PBS, individual sera were serially diluted in BSA-PBS and incubated for 2 h at 37 °C. After washing, biotinylated F(ab)₂ goat anti-mouse IgG (1:3000) (Jackson ImmunoResearch, West Grove, PA, USA) were added and incubated for additional 2 h at 37 °C following incubation with ExtrAvidin peroxidase conjugate (0.5 μ g/ml, Sigma). The reaction was visualized by adding 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, 2.5 mg/ml, Sigma) in 10% diethanolamine buffer containing 0.0025% H₂O₂. The absorbance was recorded in a spectrophotometer Spectra MaxPlus at 405 nm. The optical density (OD) values registered were related to the OD values obtained from normal (naive) mouse sera.

Statistical analyses. Values are expressed as mean \pm SEM. Values of $P < 0.05$ were considered statistically significant. Statistical tests used were Mann–Whitney, paired *t*-test, unpaired *t*-test.

Results

Histamine levels in synovial fluids and sera of patients with RA and of healthy controls

Circulating levels of histamine from patients with RA was significantly lower compared with those of healthy subjects (0.93 ± 0.16 ng/ml versus 1.89 ± 0.45 ng/ml, $P = 0.0005$) (Fig. 1). Interestingly, paired comparison of histamine levels in the blood and the synovial fluid of RA patients showed that in the joints, histamine levels were even lower (0.93 ± 0.16 ng/ml versus 0.37 ± 0.06 ng/ml, $P = 0.0006$). In contrast, there were no significant differences regarding histamine levels in SF of RA patients and of controls with non-inflammatory joint disease (0.37 ± 0.06 ng/ml versus 0.53 ± 0.12 ng/ml, ns). Comparison of circulating histamine levels of RA patients with erosive (0.92 ± 0.22) versus non-erosive (1.01 ± 0.22) RA, demonstrated that patients with erosive RA had somewhat lower levels of histamine in their blood compared with patients with non-erosive RA (not significant).

Changes of histamine levels during anti-TNF- α treatment

To assess the effect of anti TNF- α treatment on histamine blood levels, samples were collected from 10 RA

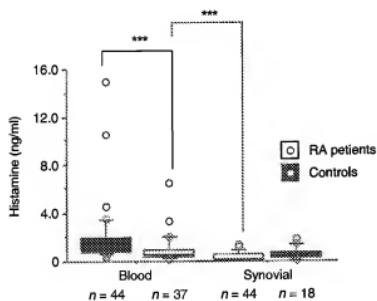


Figure 1 Histamine levels in the blood and in the synovial fluid of RA patients and controls. *** $P < 0.001$, synovial fluid versus blood in RA patients. *** $P < 0.001$, blood of RA patients versus blood of healthy blood donors.

Table 2 Impact of infliximab treatment on circulating histamine levels ($n = 10$).

	DAS28	Histamine (ng/ml)	IL-10 (ng/ml)	CRP	WBC (10^9 /ml)	Platelets (10^9 /l)	Hb (g/l)
Before infliximab	5.3 ± 1.1	0.6 ± 0.2	8.1 ± 1.4	52 ± 51	9.0 ± 4.3	355 ± 164	124 ± 15
After five infusions	3.8 ± 1.2	1.1 ± 0.2	8.4 ± 3.1	17 ± 16	7.0 ± 2.2	290 ± 129	131 ± 11
P	0.013	0.01	ns	0.049	ns	ns	ns

patients prior to the first infusion of infliximab and following 40 weeks of treatment (a total of five infusions of infliximab). The levels of histamine in circulation before and after the sixth anti-TNF- α treatment are shown in Table 2. Treatment led to a significant increase of histamine levels in the blood (0.60 ± 0.07 ng/ml vs. 1.10 ± 0.14 ng/ml, $P = 0.005$) (Fig. 2). The increase in histamine levels was inversely correlated with the DAS score ($r = -0.48$) and CRP levels ($r = -0.42$) (Fig. 3A,B). The measurement of circulating IL-10 levels before and after the anti-TNF- α treatment did not display any significant differences. Single infusion of infliximab had no obvious effect on histamine levels (mean: 0.91 ng/ml before and 1.04 ng/ml, after infusion (not significant).

Does histamine modulate inflammatory responses intra-articularly?

The histological analysis of mouse knee joints injected with 0.01 – 10 ng ($n = 24$) of histamine revealed no signs of synovitis, such as intra-articular influx of leukocytes or pannus formation. To assess if histamine eliminates inflammatory responses, we injected histamine in combination with high mobility group (box chromosomal) B1 protein (HMGB1). HMGB1 triggers the development of arthritis when administered to healthy joints [30–32]. Peptidoglycan, a component of the Gram-positive bacterial cell wall is also known to induce inflammatory response in healthy joints [33–34]. Histamine (10 ng) was co-administered to the joint cavity together with either HMGB1 (2 μ g, $n = 16$) or with peptidoglycans (10 or 50 μ g, $n = 16$) did not significantly affect the inflammatogenetic properties of these molecules. The frequency and severity of joint inflammation in mice injected with histamine/peptidoglycan mixture was similar to those found in the mice injected with peptidoglycan alone (73% versus 82%). The frequency of joint inflammation in mice injected with histamine/HMGB-1 mixture was somewhat higher than in mice injected with HMGB-1 alone (73% versus 36%) (ns).

Does stabilization of mast cell membrane affect *in vivo* inflammatory responses?

Treatment with SC had no significant effect on degree of paw swelling triggered by olive oil. Indeed no differences in paw thickness were detected between naive and SC-treatment.

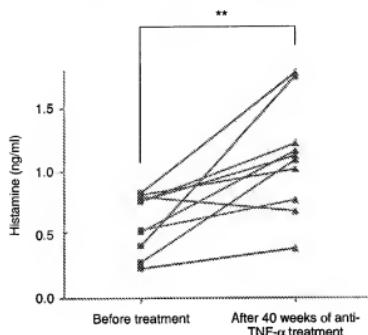


Figure 2 Increase in histamine levels in plasma of RA patients treated with infliximab ($n = 10$). ** $P < 0.01$.

ted mice. This indicates that MC inhibition does not prevent granulocyte-mediated inflammatory response. Furthermore, treatment with sodium cromoglycate had no effect on the degree of ear swelling in OXA-sensitized and challenged mice. No difference in ear thickness was detected between control and treated groups, indicating that lymphocyte/macrophage-mediated skin inflammation was not abrogated by MC inhibition. No differences in serum levels of anti-oxazolone antibodies were detected between the sodium cromoglycate treated and control groups.

Does histamine affect production of pro-inflammatory IL-6?

Mononuclear spleen cell cultures ($n = 3$) were exposed to the increasing concentrations of histamine (0–100 μ M) in the presence and absence of T-cell mitogen Con A or superantigen TSST-1. Supernatants were collected after 72 h and an analysis of IL-6 levels was performed. Treatment of splenocytes with histamine alone did not induce production of IL-6. TSST-1 and Con A-induced production of IL-6 (55 ± 4 pg/ml and 80 ± 15 pg/ml). This production was not affected by the presence of histamine in the culture medium (Table 3).

Discussion

The aims of our study were to: (a) analyse the relationship between histamine levels in the SF and the blood of RA patients; (b) evaluate the impact of a TNF- α inhibition on histamine levels in RA patients; (c) determine whether the histamine molecule itself induces inflammation in the joint cavity of healthy recipients; and (d) assess if histamine has immunomodulatory properties.

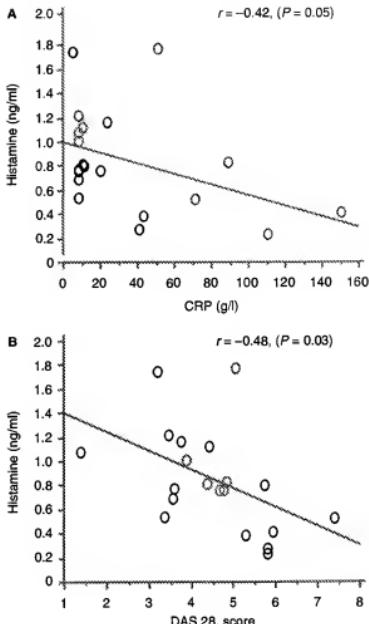


Figure 3 Circulating histamine levels of RA patients treated with infliximab are inversely related to CRP levels (A), and to the disease activity score (B).

Table 3 IL-6 production by mouse spleen cells ($n = 3$), after treatment with different concentrations of histamine.

Histamine (μ M)	Con A	TSST-1	Mock
100	63	48	16
10	47	52	12
1	108	55	13
0	80	55	12

The SD of Con A (histamine 0 μ M) was 15 pg/ml while that of TSST-1 was 4 pg/ml.

Our present data point to the fact that, within the joint cavity, histamine is actively consumed as its levels are significantly lower in the joint fluid than in the circulation of RA patients, despite the fact that the synovial cells are able to actively synthesize this molecule *in situ*. The idea of histamine consumption during inflammation

in RA patients is also supported by the observation of low histamine levels in circulation compared with healthy blood donors. We prospectively measured histamine levels in the blood of RA patients undergoing anti-TNF- α treatment. TNF- α promotes inflammatory responses that are important in the pathogenesis of RA and studies have proved that blocking TNF- α improves the course of RA [15, 35, 36]. An analysis of various inflammation markers in 10 RA patients showed that clinical improvement of symptoms was linked to the increased histamine levels in the majority of the RA patients undergoing anti-TNF- α treatment (Fig. 2). This indicates that TNF- α inhibition leads to a significant increase in histamine levels in the blood of RA patients, suggesting that histamine is more likely to be connected to the halting of the disease process in RA, on the contrary to suggestions from most previous studies.

Injection of histamine *per se* did not cause synovitis in healthy recipients. The doses of histamine were chosen based on our *ex vivo* findings in human joints. To ensure broad spectrum of histamine concentrations, up to 1000-fold differences of its intra-articular amount were used. Although histamine usually elicits strong inflammatory response, its effects are typically short lived. The outcome observed could possibly be attributed to short biological half-life of histamine [37]. Given that histamine alone failed to induce an inflammation, we combined histamine with two known pro-inflammatory substances, HMGB-1 [30, 32] and peptidoglycan [1, 34]. The question was whether histamine could affect the HMGB1/peptidoglycan-induced inflammation. Our results suggest that histamine does not influence the inflammatory effects of HMGB1 and peptidoglycan in the joint cavity.

IL-6 is a pro-inflammatory cytokine that is known to be present in high levels in the SF from RA patients [2]. It is produced by many cell types and is thought to play an important role in RA [38]. In our study, exposure of leukocytes to histamine did not have any effect on the production of IL-6. It has been shown that histamine is capable of inducing a dose-dependent IL-6 production in endothelial cells and the observed difference in results may be due to the different cell type used in our study.

Use of SC, an MC stabilizing agent, did not affect *in vivo* cell-mediated inflammatory responses. Stabilization of MC is believed to decrease their degranulation activity and thereby histamine release. SC has also been successfully used to determine the role of MC in a collagen II-induced arthritis model in mice [6, 39]. The ineffectivity of SC in the oxazolone-induced DTH reaction was surprising, given that both early and late components of this reaction are dependent on MC [29, 40]. Equivalent ear swelling responses have been found in MC-deficient and wild-type mice [41–43], although strongly reduced ear swelling in MC-deficient animals has also been observed [29]. Route of delivery could potentially have affected the

outcome of our experiment, as different methods have been used in various studies. However, our results are in agreement with one study that also examined the effect of SC on DTH reactions and came to the conclusion that it does not alter the responses significantly [43]. Administration of SC *in vivo* did not decrease the olive oil-induced inflammation. Indeed, this type of inflammation operates through the activation of neutrophils and is independent of MC and T cells [27]. In the setting of RA, we believe that properties of histamine may be mediated through its actions on neutrophils. Neutrophils release a range of proteases, hydrolases and also have the ability to produce a series of reactive oxygen intermediates (ROI), therefore having a great capability to inflict damage and cartilage degradation. It has been shown that primed and activated neutrophils in RA synovial fluid secrete above enzymes and ROI within the joint [36]. Histamine inhibits the chemotaxis of neutrophils and production of their cytokines, such as IL-1, IL-12 and TNF- α [20, 24–26, 44]. By binding to H2 receptors, histamine also inhibits phagocytic NADPH oxidase activity causing a decreased production of oxygen radicals [45]. Therefore, we suggest that histamine blocks the production of neutrophil-derived ROI, thus preventing tissue degradation in the joint. Our results show that histamine levels in the blood rise after the treatment and this might suggest that the beneficial action of TNF- α antagonists is mediated through the histamine-dependent inhibition of neutrophil extravasation.

Altogether our study points out that: (a) histamine levels are significantly suppressed in RA; (b) efficient immunomodulatory treatment reverses this suppression. These findings together with other well-known properties of histamine suggest that histamine is anti-inflammatory rather than pro-inflammatory in the setting of human RA.

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